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Partial Characterization of Proteolytic and Milk Clotting Enzymes in Sodom Apple *Calotropis procera* (Ait.) R.Br. (Asclepiadaceae) Plant

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ABSTRACT

Calotropis procera (Sodom apple) or Bomubomu in Yoruba Language has been used locally in the Northern part of Nigerian and the Fulani set-ups in African settings as curdling agent in the production of 'warankasi' (a local soft cheese) when using cow milk; this plant is also known for its medicinal importance like antidote for snake bite, sinus fistula, rheumatism, mumps, burn injuries, body pain and treatment of jaundice in India and some parts of Africa. However, this present study was undertaken to characterize the milk clotting and proteolytic enzymes in the plant with the aim of establishing the optimal conditions of the enzymes to be used for cheese and allied milk products manufacturing and protein hydrolytic processes of medicinal importance. The results obtained from this study showed that the enzyme activities were evidenced in all the plant parts extract preparations except the root that had only proteolytic activity without noticeable milk-clotting activity for the period of one hour of investigation. The optimal pH for proteolytic and milk clotting enzyme activities were observed to be 4.0 for proteolytic and 5.0 for milk clotting enzyme activities in the crude extracts using casein and powdered milk as substrates, respectively while the temperature optima for the two enzyme activities were observed to be 70°C for milk clotting and 60°C for proteolytic. Calcium ion and cysteine were found to activate the two enzymes, with maximum activation at 800 mM Ca^{2+} for proteolytic activity and 10 mM for milk clotting activity while cysteine showed maximum activation at concentration of 6.0 mM for the two enzymes as Pb^{2+} ions caused inhibition of both enzymes at the very least concentration. The results of analyzes from this work showed how the proteolytic and milk clotting enzyme activities of the plant latex could be enhanced for protein hydrolytic processes of medicinal importance and industrial production of cheese and allied milk products.

Key words: Proteolytic enzyme, milk clotting activity, optimal conditions, *Calotropis procera*

INTRODUCTION

The plant of *Calotropis procera* (of family Asclepiadaceae) has been investigated to be medicinal valuable, it is co mMonly called 'bomubomu' in South-western Nigeria where it has been used as curdling agent in local production of soft cheese amongst the Hausa-Fulani women. Though country like India used the latex as a blistering agent in traditional medicine (Nadkarni, 1976). It has also been reported that the latex caused severe skin irritation, burning sensation, swelling of the eyelids and blurring of vision if it comes in contact with the body (Matthayya, 1948; Wong, 1949; Crawford, 1958; Sugiki, 1966; Duke-Elder and Macfawl, 1972) though recovery from

these effects is spontaneous and complete which requires no specific treatment as reported by Grant (1974). Rennin or other protease enzyme has been used in the coagulation of milk resulting in the more or less specific cleavage of the 105-106 (Phenylalanine-Methionine) bond in the kappa-casein molecule which is a large peptide (65 amino acids) that is acidic in nature. It is formed from about 1/4 of the hydroxyl aminio acids (Serine, Threonine); this kappa-casein molecule is deficient in six amino acids (Cysteine, Phenylalanine, Tyrosine, Tryptophan, Histidine and Arginine). The part of the molecule has a high solvation capacity, which explains both the 'Colloid protector effect' of the kappa-casein properties and the 'coagulating mechanism'.

The protein part 'k-paracasein is hydrophobic and insoluble with a basic character, after hydrolysis by the rennin or other similar coagulating enzyme, this is the only constituent, which migrates in electrophoresis at pH 8.6 to the cathode. The enzymatic reaction occurs at various temperatures (from 0-50°C); it is not the cause of the coagulation. The presence of ionised calcium is essential; the enzyme does not coagulate a solution of whole casein (Alais and Linden, 1999). Hence, the aim of this study is to determine the proteolytic and milk clotting enzyme activities and to partially characterize the enzymes in the *Calotropis procera* plant to establish to establish how best to utilize the plant extract in their uses for cheese and allied milk products manufacturing and protein hydrolytic processes of medicinal importance.

MATERIALS AND METHODS

The plant was obtained from Aba Oyo, a farm settlement in Federal University of Technology, Akure, Ondo State Nigeria. The latex was collected in a sterile container by breaking of the leaves while the other parts of the plant were obtained by up-rooting the plant. The roots were washed with water, stem were cut into pieces and the leaves were hand-picked from the plant.

Preparation of crude enzyme extract: Exactly 5.0 g of each part of the plant (leaf, tender stem, mature stem, root and fruit) were crushed separately in mortar and extracted with 25.0 mL of 0.05 M sodium acetate buffer pH 5.5 while 1.0 mL of the latex was mixed with 5.0 mL of 0.05 M sodium acetate buffer of pH 5.5 and filtered. The filtrate was centrifuged at 3000 rpm for 15 min and the supernatant obtained was stored in the refrigerator for analysis.

Determination of milk clotting activity: Exactly 0.25 g of powdered milk was weighed into a clean test tube followed by 0.75 mL of 0.05 M sodium acetate buffer pH 5.5, the test tube was shaken until the milk dissolved and was placed in water bath for 10 min at 35°C. After which, 1.0 mL of the crude enzyme was added and the time taken for the milk to clot was taken as a measure of enzyme activity.

The unit of milk clotting activity was defined as the amount of enzyme which clotted 1.0 mL of milk solution in 0.05 M sodium acetate buffer of pH 5.5 in one minute at operating temperature of 35°C.

Effect of pH on the milk clotting enzyme activity: Exactly 0.25 g of powdered milk was weighed into a clean test tube followed by 0.75 mL of 0.05 M sodium acetate buffer at pH (1.0-13), the test tubes were shaken until the milk dissolved and were placed in water bath for 10 min at 35°C. After which, 1.0 mL of the crude enzyme was added and the time taken for the milk to clot was taken as a measure of enzyme activity.

Effect of temperature on the milk clotting enzyme activity: Exactly 0.25 g of powdered milk was weighed into a clean test tube followed by 0.75 mL of 0.05 M sodium acetate buffer in separate test tubes, the test tubes were shaken until the milk dissolved and were placed in water bath for 10 min at various temperatures 40, 50, 60, 70 and 80°C. After which, 1.0 mL of the crude enzyme was added onto each test tube at the various temperature and the time taken for the milk to clot was taken as a measure of enzyme activity.

Effect of Ca^{++} ion on the milk clotting enzyme activity: Exactly 0.25 g of powdered milk was weighed into clean test tubes followed by 0.75 mL of 0.05 M sodium acetate buffer pH 5.5 into each, the test tubes were shaken until the milk dissolved and were placed in water bath for 10 min at 35°C. After which, 1.0 mL of the crude enzyme was added. The reaction mixtures were made to contain between (0-15 mM) Ca^{++} ion concentrations, the time taken for the milk to clot was taken as a measure of enzyme activity.

Effect of cysteine on the milk clotting enzyme activity: Exactly 0.25 g of powdered milk was weighed into clean test tubes followed by 0.75 mL of 0.05 M sodium acetate buffer pH 5.5 into each, the test tubes were shaken until the milk dissolved and were placed in water bath for 10 min at 35°C. After which, 1.0 mL of the crude enzyme was added. The reaction mixtures were made to contain between (0-10 mM) cysteine concentrations; the time taken for the milk to clot was taken as a measure of enzyme activity.

Determination of the proteolytic enzyme activity: The extract was assayed for protease activity by a casein degradation method using the method of Ladd and Butler (1972). The casein solution (1%) was prepared in 0.05 M citrate phosphate buffer pH 7.5 and heat treated at 100°C for 15 min in water bath, cooled and used as substrate. The reaction mixture consists of 1.0 mL of the substrate and 1.0 mL of the extract thoroughly mixed together while the control consists of 1.0 mL of the substrate and 1.0 mL of the citrate buffer. This was incubated for 1 h at 35°C and the reaction was terminated by adding 3.0 mL of cold (2°C) 10% Trichloroacetic Acid (TCA). The tubes were allowed to stand for a minimum of 1 h at 2°C in a refrigerator to allow the undigested protein to precipitate. Thereafter, the mixtures were centrifuged at 3000 rpm for 30 min under room temperature and the absorbance of the clear supernatant was measured at 280 nm, the control was used as blank. One unit of the protease activity was defined as the amount of enzyme that liberated 1.0 μg of tyrosine under the standard assay conditions.

Effect of pH on the proteolytic enzyme activity: The reaction mixture consists of 1.0 mL of the substrate at various pHs between (1.0-13) in test tubes and 1.0 mL of the enzyme solution added to each test tube which were thoroughly mixed and incubated for 1 h at 35°C in a water-bath and the reaction was terminated by adding 3.0 mL of cold (2°C) 10% Trichloroacetic Acid (TCA) to each test tube and the procedure continued as described above.

Effect of temperature on the proteolytic enzyme activity: The reaction mixture consists of 1.0 mL of the substrate and 1.0 mL of the enzyme solution which were thoroughly mixed and incubated for 1 h at various temperatures (40, 50, 60, 70 and 80°C in a water-bath and the reactions were terminated by adding 3.0 mL of cold (2°C) 10% Trichloroacetic Acid (TCA) to each test tube at the various temperatures and the procedure continued as described above.

Effect of Ca^{++} on the proteolytic enzyme activity: The reaction mixture consists of 1.0 mL of the substrate and 1.0 mL of the enzyme solution thoroughly mixed together. The reaction mixtures were made to contain between (0-1000 mM) Ca^{++} ion concentrations in each test tube which were then incubated for 1 h at 35°C and the reaction terminated by adding 3.0 mL of cold (2°C) 10% Trichloroacetic Acid (TCA) to each test tube and the procedure continued as described above.

Effect of cysteine on proteolytic enzyme activity: The reaction mixture consists of 1.0 mL of the substrate and 1.0 mL of the enzyme solution thoroughly mixed together. The reaction mixtures were made to contain between (0-10 mM) cysteine concentrations; which were then incubated for 1 h at 35°C and the reaction terminated by adding 3.0 mL of cold (2°C) 10% trichloroacetic acid (TCA) to each test tube and the procedure continued as described above.

Statistical analysis: The results from these analyses were obtained in triplicates and the means determined. Various formulae were used to calculate the milk clotting and proteolytic enzyme activities which were then constructed to obtain the plots using Microsoft Office Excel 2007.

RESULTS

The results of analyses showed that all the parts of the plant possessed relative proteolytic activity which ranged from 21.4-100%; the results obtained for the latex and root extracts were significantly higher than those obtained for all other parts of the plant while the relative milk clotting activity ranged from 2.5-100% with the latex having a significantly higher values than other parts. Though the root had a relative milk clotting activity of zero but second to the latex with 62.3% in relative proteolytic activity. The result also indicated that the relative milk clotting activity was conspicuously low in all the plant parts with the exception of the latex as obtained from Fig. 1. The results also showed that the enzyme activities were optimal at acidic pH 4.0 and 5.0 for proteolytic and milk clotting enzyme activities, respectively. However, there was complete lost of milk clotting activity at pH 1.0-4.0 and 8.0-13.0 while the proteolytic activity was observable even at pH 1.0 but completely lost between pH 9.0 and 13.0 as seen from Fig. 2; while optimal temperatures were obtained at 60 and 70°C for proteolytic and milk clotting activities, respectively, though activity was still observable at 80°C (Fig. 3). Ca^{++} ion and cysteine caused activation of these enzymes while Pd^{++} ion caused inhibition even at very least concentration of 1.0 mM in the

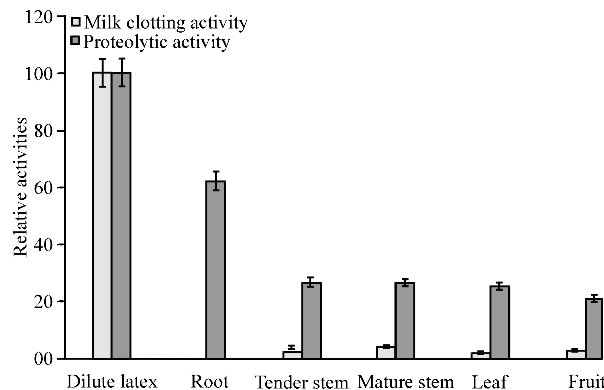

Fig. 1: Milk clotting and proteolytic enzymes activities of *Calotropis procera* plant Mean±SE

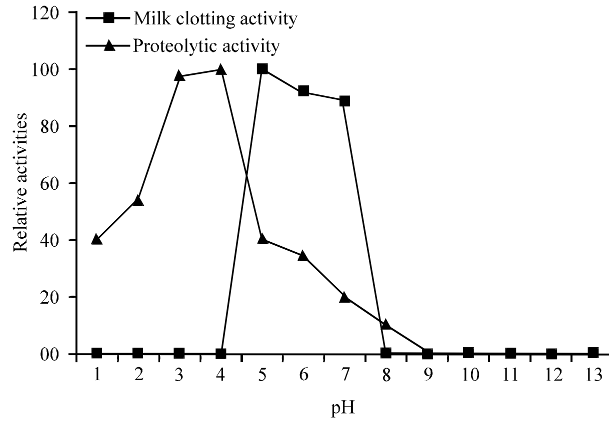

Fig. 2: Effect of pH on milk clotting and proteolytic enzymes activities of *Calotropis procera* latex

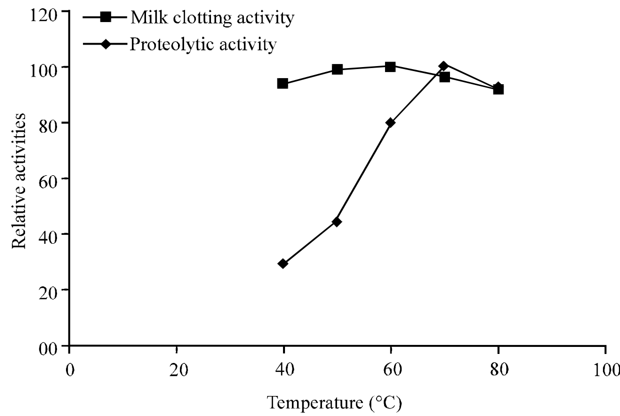

Fig. 3: Effect of temperature on milk clotting and proteolytic enzymes activities of *Calotropis procera* latex

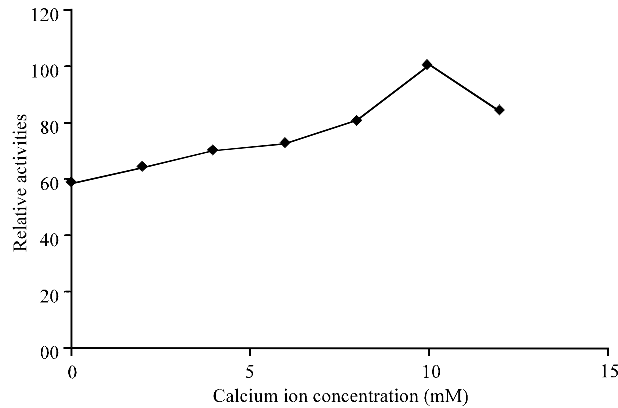

Fig. 4: Calcium ion activation of milk clotting enzyme activity of *Calotropis procera* latex

two enzymes (Fig. 4-7). The activation of milk clotting and proteolytic enzymes activities were observed until 10 mM and 800 mM calcium ion concentration, respectively. While activation by

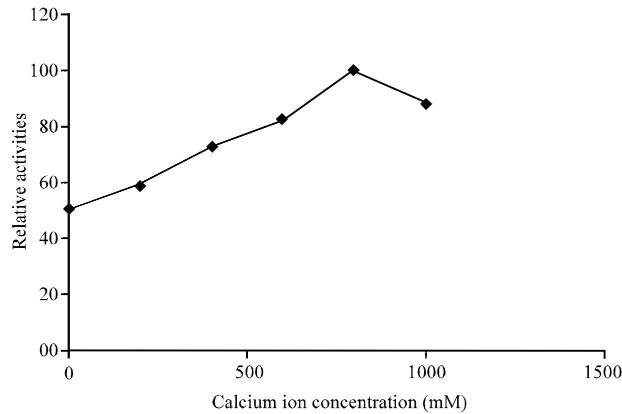

Fig. 5: Calcium ion activation of proteolytic enzyme activity of *Calotropis procera* latex

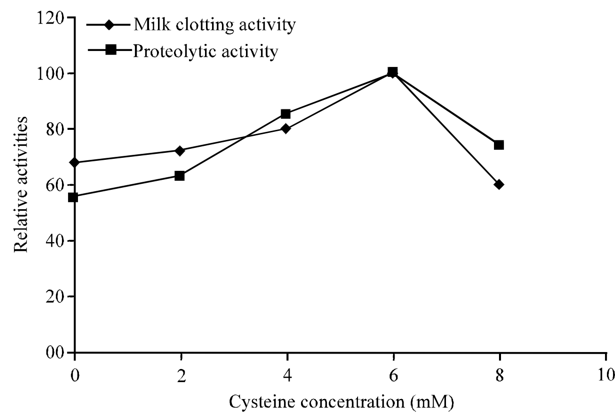

Fig. 6: Activation effect of cysteine on milk clotting and proteolytic enzymes activities of *Calotropis procera* latex

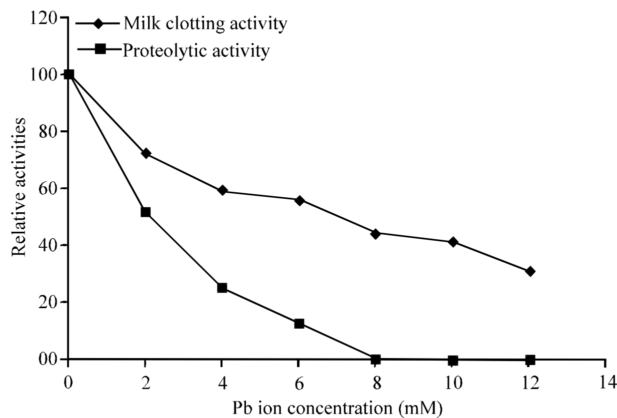

Fig.7: Inhibitory effect of Pb^{++} concentration on milk clotting and proteolytic enzymes activities

cysteine was observed until 6.0 mM concentration before decline in activities for both enzymes. The proteolytic activity was completely lost at Pd^{++} ion concentration of 8 mM, while the milk clotting was still retained at 12 mM concentration of Pd^{++}.

DISCUSSION

The milk clotting and proteolytic enzymes relative activities of the various parts of *Calotropis procera* were presented in Fig. 1. All the plant parts produced milk clotting activity with exception of the root having a zero relative activity as it did not show any observable clotting ability within the one hour assay time. The milk clotting enzyme activity of the plant decreased in the order from the latex, tender stem, mature stem, fruit and leaf. However, it has been reported that the leaf of the plant had been used as clotting agent in the production of the local soft cheese (warankasi) by Ihekoronye and Ngoddy (1985). Similarly, the proteolytic enzyme activities of the plant parts from Fig. 1 revealed the ability of the plant parts to hydrolyze casein, suggesting that proteolytic enzyme is widely distributed in all parts of the plant with the latex exhibiting highest enzyme activity followed by root, tender stem, mature stem, leaf and fruit. However, the tenderness or maturity of the stem however had very little or no effect on the milk clotting and proteolytic enzyme activities as observed from the study. In general, our findings agreed with the observations of Dahot *et al.* (1990) who screened some Pakistani plants of tropical origin for both milk clotting and protease activities; however, the milk clotting and protease activities reported in this work is far higher than that reported by Guiama *et al.* (2010) for nine species of *Solanum berries*. The result of this study with latex having the highest milk clotting and proteolytic enzyme activities however corroborate that reported earlier on that latex contained calotropain and some other cardiac glycosides and several proteinases (Abraham and Joshi, 1979a, b); as also supported by the findings of Atal and Sethi (1962) that proteolytic activity of the calotropain from *Calotropis procera* was found to be greater than that of papain, ficin, or bromelain.

The ethno medicinal importance of various parts of this plant which might not be unconnected with some of these proteases have also been studied and reported by other researchers, (Verma *et al.*, 2010; Behl *et al.*, 1966; Morton, 1962; Blohm, 1962; Nadkarni, 1976) also reported the latex to be irritant, caustic, and depilatory when applied to the skin.

The observed pH optimum (Fig. 2) for the proteolytic enzyme activity using casein as substrate was 4.0 while that of milk clotting enzyme activity using powdered milk as substrate 5.0 for the latex of *Calotropis procera*. This optimum pH of 5.0 for the proteolytic enzyme activity observed was similar to that reported for papain using gelatin as substrate, while the pH optimum of 7.5 was reported for proteolytic activity using albumin as substrate, Vanoss (1972). Hence optimum pH is dependent of the substrate used. The temperature optimum (Fig. 3) for the proteolytic and milk clotting enzymes activities in this study were obtained to be 60 and 70°C, respectively though the enzymes still retain activities at 80°C.

It was also observed in this study that calcium ions and cysteine caused activation on both milk clotting and proteolytic enzyme activities is shown from the results (Fig. 4-6) with maximum activation at 10 mM Ca^{2+} for the milk clotting enzyme and at 800 mM for the proteolytic enzyme activity and 6.0 mM cysteine which suggest that Ca^{2+} ions and cysteine will be required in the milk clotting and proteolytic assays of the enzymes as Pb^{2+} ions caused inhibition of both enzymes at the very least concentration (Fig. 7).

CONCLUSION

Crude extracts from different parts of the sodom apple plant contained milk clotting and prteolytic enzyme activities which can be used in the local production of cheese and can also be exploited for other industrial and medicinal uses. The plant can also be said to contain thermo-stable proteases which can be utilized for some specific uses. Further studies on

characterization and properties of the purified enzyme and possibly the mechanism of milk coagulation from these plants would be interesting and this will be reported in the near future.

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